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Isoforms of cellular fibronectin and tenascin in amniotic fluid

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Abstract

Amniotic fluid (AF) obtained from second trimester pregnancies presented extradomain (ED) A, B and an oncofetal (onc-f) domain containing isoforms of cellular fibronectin (cFn) in Western blotting of gelatin-bound polypeptides and directly of AF. Western blotting after sequential immunoprecipitation suggested at least three Fn molecules: one containing EDA and the onc-f domain and another minor component distinctly containing all the domains, and a third one only containing EDA. The immunoblotting results for EDA-cFn and onc-f-cFn were closely similar to that for total Fn, whereas in plasma samples of normal and pregnant women only traces of EDA-cFn and onc-f-cFn, but no EDB-cFn, were found. Western blotting of AF also indicated the presence of three isoforms of tenascin (Tn), M_r 190,000 and 280,000 polypeptides earlier found in many cells, and a M_r 200,000 polypeptide, novel for AF and not present in plasma. The results suggest a novel extracellular matrix polypeptide composition for AF.

Key words: Amniotic fluid; Fibronectin; Plasma; Tenascin; Isoform; Extracellular matrix

1. Introduction

Fibronectins (Fn's) are a family of high molecular weight glycoproteins present in extracellular matrix (ECM) fibrils and soluble in various body fluids which play a role in several biological phenomena [1]. In contrast to plasma Fn, produced by hepatocytes and found soluble in body fluids and deposited in ECM [1], cFn's contain extradomain (ED) sequences generated by differential splicing of single mRNA [1,2] or present a distinct pattern of glycosylation in an onc-f (oncofetal) isoform [3]. Among the cFn's, EDA-cFn appears to be a minor component in plasma [4,5] but more distinctly present in bronchioalveolar lavage fluid [6] and amniotic fluid (AF) [7], the latter also containing onc-f-cFn [8]. It has been proposed that AF-Fn is locally produced rather than derived from plasma [9,10]. Since then, distinct differences have been found in glycan residues of AF [11–13] and placental Fn, as well as in their gelatin-binding properties [12,14–16] when compared to plasma Fn. Tenascin is a novel ECM protein sharing sequence homology to Fn but presenting opposite functions [17,18], and is expressed during organogenesis and carcinogenesis [17–19]. Here we have studied in detail the presence of Fn isoforms and Tn in AF by using isoform-specific monoclonal antibodies (MAb's).

2. Materials and methods

2.1. Source and preparation of AF

AF was derived by amniocentesis performed for prenatal chromosome analysis at 15–16 weeks of gestation. AF was centrifuged at 1,000 × *g* for 10 min to separate the cells. Small quantities of the supernatant were used for Western blotting experiments, either directly or after gelatin–Sepharose (Pharmacia, Uppsala, Sweden) affinity chromatography [20]. Fresh samples of plasma were taken by routine methods gfrom normal and pregnant women.

2.2. Western blotting and immunoprecipitation

For some Western blotting experiments, AF or plasma was first subjected to immunoprecipitation using the MAb, DH1, to EDA-cFn [4] as described [21], MAb FDC-6 to onc-f-cFn [8] or MAb 100EB2 for all isoforms of Tn [22]. SDS-PAGE was done using 6.5% slab gels under reducing conditions [23]. Then, the polypeptides were transferred onto nitrocellulose membranes [24] and either stained for protein or immunostained. For the latter purpose the following MAb's were used: DH1, BC-1 for EDB-cFn [25]; FDC-6, BF12 for total Fn [4]; BC-4 [26]; and 100EB2 [22]. For immunostaining, the membranes were first incubated overnight in 3% bovine serum albumin-phosphate buffered saline solution and then exposed to the MAb's followed by the peroxidase-coupled second antibody (Dako, Glostrup, Denmark).

2.3. Characterization of the MAb 100EB2

The MAb 100EB2 was raised against Tn from spent culture medium of human embryonal fibroblasts as described [22]. To further characterize this MAb, it was mapped by ELISA to specific domains of Tn by G. Briscoe and H.P. Erickson (Duke University, Durham, NC) [27]. For this purpose, bacterial and mammalian expression proteins corresponding to specific Tn domains [27] were coated on 96-well plastic dishes (Falcon) at 4°C at protein concentration of 5 μg/ml overnight. The wells were washed and hybridoma supernatant containing the antibody to be tested was added to the wells for 2 h. The wells were washed and bound antibody was detected by peroxidase-conjugated secondary antibody against mouse IgG.

The MAb 100EB2 gave a positive reaction in wells coated with HxB.L (the largest splice variant of human Tn) TnFn1-5 and TnFn3-5

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(see [27] for domain nomenclature). It gave no reaction with any of the other expression proteins tested, including TnFn1-3. Therefore, this MAb binds to an epitope in the Fn-III domain number 4 or 5.

3. Results and discussion

3.1. Fn isoforms and Tn in plasma

We first studied the presence of Fn isoforms in plasma samples taken from normal and pregnant women. Immunoblotting experiments of SDS-PAGE separated polypeptides from gelatin–Sepharose affinity chromatography suggested that, while the amount of total Fn, as revealed with the MAb BF12, was high in both samples (Fig. 1 panels A,B lane 1), only traces of EDA-cFn (Fig. 1A,B, lane 2) and, in contrast to a previous study [8], also onc-f-cFn (Fig. 1A,B, lane 3) could be detected. Even after excessive loading EDB-cFn was not detected (Fig. 1A,B, lane 4). Due to a high protein concentration direct immunoblotting experiments of plasma samples were unsuccesful. Immunoprecipitation experiments of both plasma samples with the MAb 100EB2 revealed a doublet of Tn polypeptides of M_r 280,000 and 190,000 (Fig. 1A,B, lane 5).

3.2. Fn isoforms in AF

Immunoblotting experiments of gelatin–Sepharose-bound polypeptides with the MAb's to Fn isoforms revealed that in five different samples of AF, EDA (Fig. 2A), EDB (Fig. 2B) and the onc-f domain (Fig. 2C) containing isoforms could be detected. Although the reactions between individual samples varied, the reaction for EDB was consistently weaker than that for other cFn's. As Fn from AF has been reported to have a reduced affinity to gelatin [12,14,16] we also made direct immunoblottings of AF samples. These experiments revealed distinct reactions for total Fn (Fig. 3, lane 1), EDA-cFn (Fig. 3, lane 2), onc-f-cFn (Fig. 3, lane 3) and a weaker reaction for EDB-cFn (Fig. 3, lane 4). All these experiments show that, in contrast to plasma, cFn's are a prominent part of AF Fn.

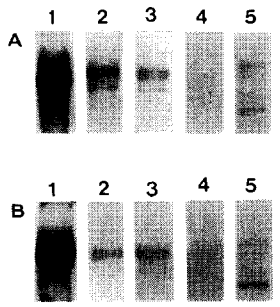

Fig. 1. Western blotting of SDS-PAGE-separated gelatin-bound polypeptides from plasma of pregnant (A) and normal (B) women for Fn with the MAb's, BF12 for total Fn (lane 1), DH1 for EDA-cFn (lane 2), FDC-6 for onc-f-cFn (lane 3) and BC-1 for EDB-cFn (lane 4). Immunoprecipitation of both plasma samples with MAb 100EB2 for all isoforms of Tn is shown in lane 5.

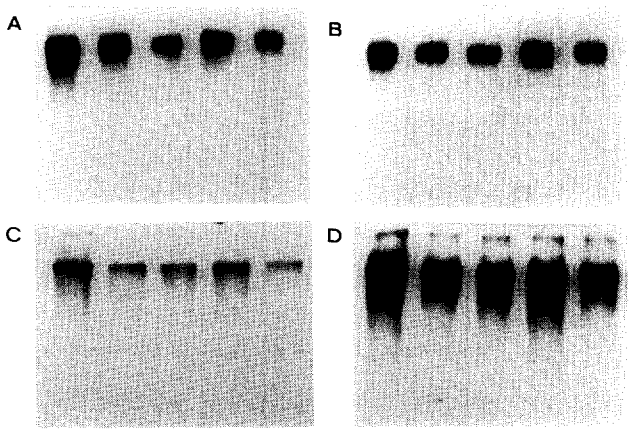

Fig. 2. Western blotting of SDS-PAGE-separated gelatin-bound polypeptides from 5 different AF samples for EDA-cFn (A), onc-f-cFn (B), EDB-cFn (C) and total Fn (D).

The composition of Fn isoforms in AF was further investigated by immunoprecipitation experiments with the MAb DH1 of AF subjected to Western blotting after SDS-PAGE. These experiments revealed that the EDA-cFn immunoprecipitate gave prominent reactions for total Fn (Fig. 4, lane 1), EDA-cFn (Fig. 4, lane 2) and for onc-f-cFn (Fig. 4, lane 3) but that for EDB-cFn was much weaker (Fig. 4, lane 4). On the other hand, a similar experiment with the MAb, BC-1, showed that EDB-cFn contained both EDA-cFn and the onc-f-cFn epitopes (not shown). Then we studied the Fn profile of AF after depletion of the onc-f-cFn by two subsequent immunoprecipitations with the MAb, FDC-6. Western blotting of the first precipitate revealed prominent reactions for the MAb's, FDC-6 (Fig. 5, lane 1) and DH1 (Fig. 5, lane 2), and a weaker reaction for the MAb, BC-1 (Fig. 5, lane 3). The supernatant after two precipitations was subjected to gelatin–Sepharose and analyzed for cFn isoforms: onc-f-cFn (Fig. 5, lane 4) and EDB-cFn (not shown) could no longer be demonstrated, indicating that

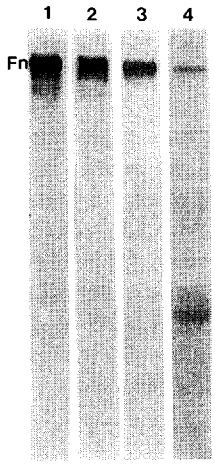

Fig. 3. Western blotting of AF for Fn. Total Fn (lane 1), EDA-cFn (lane 2) onc-f-cFn (lane 3) and EDB-cFn (lane 4).

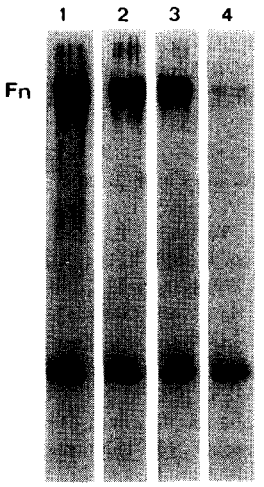

Fig. 4. Immunoprecipitation of AF with MAb DH1 for EDA-cFn and Western blotting of SDS-PAGE-separated polypeptides for total Fn (lane 1), EDA-cFn (lane 2), onc-f-cFn (lane 3) and EDB-cFn (lane 4).

all onc-f-cFn had been removed together with EDB-cFn. However, antibodies to EDA-cFn (Fig. 5, lane 5) and total Fn (Fig. 5, lane 6) still gave immunoreactive bands. These results suggest that in AF there are three distinct cFn's: a major polypeptide containing both EDA and the onc-f domain, another minor polypeptide containing all three epitopes, and a third containing only EDA. Although the results do not allow quantitative evaluation they suggest that the amount of pFn, lacking cFn epitopes, is low in AF, while the opposite appears to be the case with plasma Fn.

3.3. Tn in AF

Immunoblotting experiments with the MAb's to Tn polypeptides were also done with AF samples. MAb

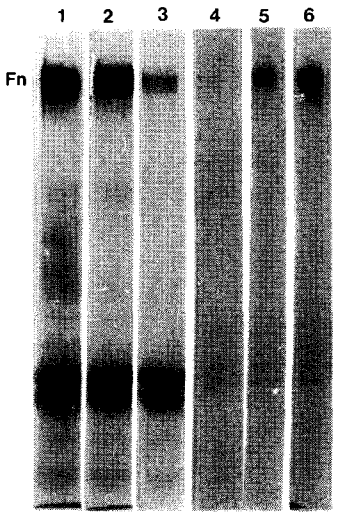

Fig. 5. Immunoprecipitation of AF with MAb FDC-6 and Western blotting for onc-f-cFn (lane 1), EDA-cFn (lane 2) and EDB-cFn (lane 3). Western blotting of gelatin–Sepharose-bound polypeptides after the second immunoprecipitation with MAb FDC-6 for onc-f-cFn (lane 4), EDA-cFn (lane 5) and total Fn (lane 6). Note the lack of reaction in lane 4, and detectable reactions in lanes 5 and 6.

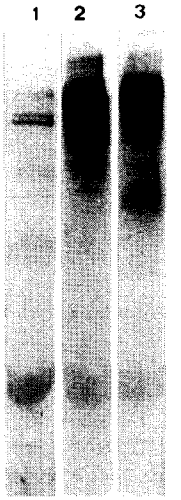

Fig. 6. Western blotting of AF with MAb 100EB2 to tenascin (lane 1). BF12 for total Fn (lane 2) and DH1 for EDA-cFn (lane 3).

100EB2 reacted with a polypeptide of M_r 280,000 and a doublet of M_r 190,000 and 200,000 polypeptides (Fig. 6, lane 1). The same polypeptides could also be revealed with MAb BC-4 reacting with the amino-terminal part of Tn [26] and hence binding to all Tn polypeptides (not shown). For comparison, lanes 2 and 3 in Fig. 6 show reactions for total Fn and EDA-cFn in the same sample, respectively. Tn is a newly described ECM polypeptide sharing sequence homology with Fn but apparently displaying opposite functions [17]. The M_r 280,000 and M_r 190,000 polypeptides appear to correspond to those by a variety of mesenchymal cells in vitro [21,26] and are also produced into the ECM of cultured amnion epithelial cells [21]. The distinct M_r 200,000 Tn polypeptide may be a novel Tn variant and not a product of proteolysis as MAb BC-4, also detecting the polypeptide, reacts with the protease-susceptible amino-terminal part of the molecule [28,29].

During recent years much interest has been devoted to AF Fn and its properties in comparison to plasma Fn. While a few recent studies have suggested that EDA-cFn [7] and onc-f-cFn [8,30] might be present in AF, the present results suggest that AF contains three distinct locally produced cFn isoforms together with three Tn polypeptides.

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